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(57) Abstract

Disclosed is the use of bacteria endowed with arginine deiminase to induce apoptosis and/or reduce an inflammatory reaction, and pharmaceutical or dietetic compositions containing such bacteria. Also inclosed is a strain of *Lactobacillus brevis* highly endowed with arginine deiminase.

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Use of bacteria endowed with arginine deiminase to induce apoptosis and/or reduce an inflammatory reaction and pharmaceutical or dietetic compositions containing such bacteria

The present invention concerns the use of bacteria endowed with arginine deiminase to induce apoptosis and/or reduce an inflammatory reaction, and pharmaceutical or dietetic compositions which contain such bacteria. The invention also concerns a strain of *Lactobacillus brevis* which is highly endowed with arginine deiminase.

The balance between the cell population in an organism can be controlled by way of regulating the rate of proliferation or differentiation or death of the constituent cells (Collins, M. K. L. et al, A. Trends Biochem. Sci. 18:307, 1993). Cell death during embryogenesis, metamorphosis, hormone-dependent tissue atrophy and the normal turnover of the tissues is referred to as "programmed cell death". For the large part that event takes place by way of "apoptosis", a process which is characterised by condensation and segmentation of the nucleus, condensation and fragmentation of the cytoplasm and often fragmentation of the chromosomic DNA into nucleosomal units (Schwartz, L. M. et al Immunol. Today 14:582, 1993). Apoptosis in the development of vertebrates often occurs when the cells do not receive the extracellular survival signals necessary to suppress an intrinsic cell suicide programme: the survival factors can be produced by the surrounding cells of different type (paracrine

mechanism) or of the same type (autocrine mechanism). Apoptosis occurs during embryonic development in particular in complex organs where a given cell sub-population is killed. For example many neurons move in the brain during development, just as auto-reactive T lymphocytes are eliminated in the interior of the thymus. In an adult apoptosis occurs in particular in tissues which are subjected to reversible expansion as in the hormone-dependent cells of the breast and the prostate gland after removal of the hormone or following cytokine-dependent expansion of the haemopoietic cells of the bone marrow.

The modifications which occur in the cell in the course of apoptosis have been widely studied and described (Cohen, J. J. et al Lab. Clin. Med. 124:761, 1994). Apoptosis is clearly different from necrosis which corresponds to the modifications which occur when cell death derives from cell damage. In necrosis in fact the damaged cells swell up and burst, releasing their intracellular content which is toxic in relation to other cells of the tissue, and triggering off an inflammatory response. In contrast phagocytosis of the apoptotic bodies is so fast as not to induce dispersion of the cellular contents in the extracellular space which otherwise would cause perilesional phlogosis typical of necrosis.

Recent experimental evidence indicates that alterations in cell survival contribute to the pathogenesis of many human diseases including cancer, viral infections, auto-immune diseases, neurovegetative disorders and AIDS (Thompson, C. B. Science 267:1456, 1995). A treatment aimed at specifically altering apoptosis can have the potential to modify the natural progression of some of those diseases. Both chemotherapy agents and radiation induce the death of tumour cells primarily causing damage to the DNA which in turn causes cell suicide. In addition many tumours conserve some of the physiological cell death control systems which are characteristic of the cells from which they originate. For example cancer of the prostate and cancer of the breast are respectively androgen- and oestrogen-dependent. Therefore anti-androgenic therapy in the

treatment of cancer of the prostate gland or removal of oestrogens by means of anti-oestrogens such as tamoxifen in the course of breast cancer are fundamental and universally accepted procedures. Both those methods induce apoptosis in the tumour cells which are otherwise respectively dependent for their survival on androgens or oestrogens. In addition the beneficial effects of glucocorticoids observed in subjects with lymphoidal leukaemia can be attributed to the induction of apoptosis; other substances used for chemotherapy of cancer such as cyclophosphamide, metotrexate, etoposide and cisplatin induce apoptosis of tumour cells (Thatte, U. et al Apoptosis. Drugs 54:511, 1997).

Previous studies have shown that lactic bacteria present in foods and/or in dietetic/pharmaceutical formulations can cause transitory colonisation of the intestine and have beneficial effects. Survival during the intestinal transit or adhesion to the epithelium seem to be important for modifying the immune response of the host (Schiffrin, E. J. et al Am. J. Clin. Nutr. 66: 515S, 1997). The potentially beneficial effects of lactic bacteria include protection from enteric infections, stimulating the secretion of IgA, and inhibition of the growth of intestinal carcinoma, strengthening the activity of IgA, T-cells and macrophages (Perdigon, G. et al J. Dairy Sci. 78:1597, 1995). *In vitro*, lactic bacteria have revealed a capacity to stimulate the production of alpha TNF, interleukin (IL)-6 and IL-10 on the part of human mononuclear cells, even to an extent greater than that revealed when using lipopolysaccharide (LPS) as a stimulating agent, confirming a potentiating action on non-specific immunity of the host (Miettinen, M. et al Infect. Immun. 64:5403, 1996). Still *in vitro*, lactic bacteria have demonstrated a capacity to absorb mutagenic substances present in cooked foods, confirming the observation that the administration of lactobacilli in man reduces the excretion of mutagenic substance after the ingestion of fried meat and thus the risk of cancer of the colon (Lidbeck, A. et al Eur. J. Cancer Prev. 1:341, 1992). Experiments conducted with fermented milk with *Bifidobacterium infantis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*,

Lactobacillus acidophilus, or *Lactobacillus paracasei* on the growth of breast tumour cells MCF7 have demonstrated that the various fermented milks are capable, even if to varying degrees, of inhibiting the growth of tumour cells. The anti-proliferative effect cannot be correlated to the presence of the bacteria in the fermented milk, to the milk or to the fractions thereof; the hypothesis is for the presence of a soluble compound produced *ex novo* from the lactic bacteria during fermentation of the milk or microbial transformation of some components of the milk into a biologically active form (Biffi, A. et al Nutr. Cancer 28:93, 1997).

Many micro-organisms use arginine as a source of carbon, nitrogen and energy. Arginine deiminase transforms arginine in the presence of water into citrulline and ammonia. That enzymatic procedure has been encountered in a variety of pathogenic or potentially pathogenic bacteria such as *Pseudomonas* sp and *Bacillus* sp, and in some types of mycoplasms. It has been demonstrated that this system plays a part in oral ecology, in protecting less acid-tolerant organisms during the fall in the pH to 4, or even lower values, in dental plaque, during glycolysis caused by bacteria which are more resistant to acidity (Curran, T. M. Appl. Environ. Microbio. 61:4494, 1995).

Studies have been conducted on arginine deiminase which can be obtained from mycoplasms, to be used as a cure for cancer (Takaku, H. et al Jpn. J. Cancer Res. 1:840, 1995). Mycoplasms are micro-organisms which are similar to bacteria which, unlike the latter, lack a cell wall and the genome of which is around 1/6 of that of *E. coli*; however they can be pathogenic for man, animals and for plants and in addition they cannot be easily handled due to the absence of a cell wall. Purification was thus implemented in respect of the enzyme arginine deiminase which can be obtained from mycoplasms, which behaves like an immunogen and which is not free from undesired effects if used *in vivo* (McGarrity, J. G. et al US-A-5 372 942). Other micro-organisms endowed with arginine deiminase (such as for example *Pseudomonas* sp and

Bacillus sp) cannot be used by virtue of their potential pathogenicity and pyrogenicity.

We have now surprisingly found that some bacteria are rich in arginine deiminase, in particular some Gram-positive bacteria and some Gram-negative bacteria, and also some strains of lactic bacteria, in particular of the species *Lactobacillus brevis* or *Lactobacillus fermentum*, more particularly the strain of *Lactobacillus brevis* referred to as CD2 deposited with the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany, under the access number DSM 11988, are capable of inducing apoptosis and can therefore be used for prevention or therapy in respect of clinical situations characterised by insufficient or absent apoptosis or by inflammation.

The above-mentioned bacteria have surprisingly shown an arginine deiminase capable of inducing apoptosis and they can be used as such or after suitable lyophilisation or also after sonication. Indeed in accordance with the present invention the bacteria in question can be live or sonicated and the level of concentration can fluctuate from $1x10^1$ CFU to $1x10^{13}$ CFU per gram of composition, according to the desired effect and the amount of arginine deiminase which they have. The same bacterial strains can be used to reduce or terminate an inflammatory reaction caused by nitric oxide (NO). NO which is synthesised from L-arginine by means of nitric oxide synthase (NOS) is an intra- and intercellular messenger with numerous biological actions. Alterations in the level of synthesis of NO are at the basis of numerous other physiopathological conditions such as arterial hypertension, renal insufficiency, septic shock, vasodilation induced by hypoxia, vasospasm resulting from subarachnoid haemorrhage, neuronal destruction in vascular infarction and other neurodegenerative conditions, chronic inflammatory pathologies, anaphylaxis and immunodeficiency. Arginine deiminase converts arginine into citrulline and NH_3 without the production of nitric oxide and can thus have an anti-inflammatory and curative or remedial

effect, for example in intestinal malabsorption and pancreatic insufficiency with modulation for example of the metabolic and/or nutritional state of the subject. An effect which is referred to by way of non-limiting example can be that of reducing the levels of oxalates and/or phosphates in blood and urine.

Non-limiting examples of diseases or disorders which can be treated and/or prevented by using bacteria which are rich in arginine deiminase are tumours in general in particular colon-rectal cancer, cancer of the liver, gliomae, neuroblastomae, squamocellular oral carcinoma, lymphoid tumours, cancer of the prostate gland, cancer of the bladder, cancer of the breast, cancer of the pleura and the peritoneum, serious myasthenia, systemic lupus erythematosus, and other auto-immune diseases including those of the thyroid, diseases characterised by acute and/or chronic inflammatory processes, bronchial asthma, intestinal inflammatory diseases, gastrites, duodenites, gastric ulcers, duodenal ulcers, pneumonias and pleurisies, infections from adenovirus, baculovirus and in general supported by a viral agent, diseases characterised by acute and/or chronic inflammatory and/or degenerative processes of the central and/or peripheral nervous system, pancreatites, endomyocardites and ischaemic damage (myocardiac, retinal, cerebral and renal), urolithiases, nephrocalcinoses, hyperoxaluria, hyperphosphaturia, nephroalteration in the systemic and/or district arterial and/or venous pressure such as portal hypertension, vaginoses and vaginites, procto-haemerrhoidal inflammations, prostates, sinusites and otites, conjunctivites, gingivites, periodontopathy, anaphylactic phenomena and immunodeficiencies.

Such micro-organisms which are rich in arginine deiminase can be used individually or in combination with each or with other lactic bacteria such as *Lactobacillus acidophilus*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus catenaforme*, *Lactobacillus cellobiosus*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus jensenii*, *Lactobacillus*

leichmanii, *Lactobacillus minutus*, *Lactobacillus plantarum*, *Lactobacillus rogosae*, *Lactobacillus salivarius*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, *Bifidobacterium eriksonii*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium plantarum*, *Bifidobacterium pseudo-catenulatum*, *Bifidobacterium pseudolongum*, *Streptococcus lactis*, *Streptococcus raffinolactis*, *Streptococcus thermophilus*, *Acidaminococcus fermenta*, *Cytophaga fermentans*, *Rhodoferax fermentans*, *Cellulomonas fermentans* and *Zymomonas mobilis*.

Still in accordance with the present invention the bacteria can be used in association with arginine deiminase, sphingomyelinase or other enzymes, with cortisone, anti-inflammatory, immuno-modulant, cytostatic, immunological, endocrinological, vascular, anaesthetic, and vasodilatory drugs, growth factors, cytokines, ceramides, vitamins and minerals, lipids, amino acids and carbohydrates, formulations for enteric use and dietetic, prebiotic or probiotic supplements, and with excipients commonly used in the pharmaceutical industry or in the foodstuffs/dietetic field. The preferred form of administration is orally but it is not limitative in relation to possible topical, rectal, nasal or parenteral administration. The composition of the invention will thus be in the form of pills or tablets, capsules, globuli, suppositories, emulsions, suspensions, stick-on plasters, creams, ointments, sprays, collyria, collutoria or dentifrices.

The following examples which are set forth by way of non-limiting illustration will illustrate the present invention in greater detail.

Example 1

Induction of apoptosis in various cellular systems

Cells used:

1. Normal:

PBL (human peripheral blood lymphocytes)

HS27 (normal human fibroblasts)

HaCaT (eternalised normal human keratinocytes)

2. Tumoral:

Jurkat (human T leukaemia)

P815 (murine mastocytoma)

J744 (murine tumoral macrophages)

The cells were cultivated in suitable medium with serum (10%) at 37°C (5%CO_2) for 18-72 hours in the presence or absence of sonicated preparations in buffered solution of phosphates (PBS) of *L. brevis* (final concentration in the cellular suspensions: 100 mg/10 ml). At the end of the incubation operation the cells were counted and vitality determined on the basis of exclusion of the dye tripan blue. Possible induction of death by apoptosis in the cells treated with the bacteria was determined on the basis of:

- morphology under an optical microscope after colouring with haematoxylin/eosin.

- colouring with acridine orange/ethidium bromide detected by means of fluorescence microscopy and cytofluorimetry, and

- detection of DNA laddering by means of agarose gel electrophoresis of the DNA.

Table 1

Apoptosis (%)

	Controls	*L. brevis* (CD2)
PBL	0	0
HS27	0	0
HaCaT	0	0
Jurkat	2-3	15-20
P815	0	1-3
J744	0	1-3

The above-reported results indicate clearly that treatment for 14-18 hours with sonicated bacteria of the invention determines the induction of significant levels of apoptosis in tumoral cells, while not giving rise to any effect on the normal cell systems analysed.

Example 2

Demonstration of the presence of arginine deiminase in bacterial strains

The activity of arginine deiminase in some bacterial strains was determined on the basis of conversion in an aqueous solution of radio-marked arginine into citrulline and NH_3. The presence of suitable inhibitors (L-N-nitro-arginine methyl ester HCl and L-valine) capable of specifically inhibiting other enzymes which effect conversion of the arginine (nitric oxide synthase and arginase respectively) made it possible to attribute the enzymatic activity determined to the arginine deiminase and not to other enzymes. In addition the use of a specific inhibitor in respect of arginine deiminase (formamidine) made it possible to confirm the soundness of the results.

Table 2

Activity of arginine deiminase (expressed as pmol of radioactive citrulline produced/mg bacterial proteins per minute

Bacterial strain	pmol citrulline/mg proteins/min
L. brevis CD2	6.72
L. fermentum	0.46
L. casei	0.13
L. acidophilus	0.002
L. plantarum	0.05
B. bifidum	0.03
S. thermophilus	0.020

Bacterial strains are considered as useful for the purposes of the present invention, which have values of greater than 0.1 pmol citrulline/mg bacterial proteins/min.

The presence of inhibitors of nitric oxide synthase (L-NAME, L-nitromonomethyl arginine) or arginase (L-valine) did not in any way influence the enzymatic activity in regard to conversion of arginine to citrulline, thus making it possible to attribute the generation of citrulline observed with the various bacteria to the arginine deiminase. In addition the absence in the analysis system of calcium and calmodulin which are indispensable in terms of the activity of the constituent nitric oxide synthase did not in any way modify the activity in terms of conversion of the arginine on the part of bacteria, further confirming that the enzyme responsible for the latter is arginine deiminase.

The results set out hereinafter demonstrate that the activity of arginine deiminase encountered in the bacteria in question was further capable of completely inhibiting both the activity of constituent nitric oxide synthase (NOS) and that of inducible NOS, probably because the presence thereof involves deprivation of the substrate (arginine) of the various forms of nitric oxide synthase. For that purpose rat cerebellum extracts and rat peritoneal macrophages

stimulated *in vitro* with lipopolysaccharide of *E. coli* (100 mg/ml) and interferon (100 U/ml) respectively were used as positive controls for constituent NOS and for inducible NOS.

Table 3

Activity of constituent and inducible nitric oxide synthase and arginine deiminase

Sample	Citrulline (pmol/5 microlitres)
Cerebellum	0.24
Cerebellum + L-NAME	0.01
Cerebellum + calcium chelating agent (EGTA)	0.01
Cerebellum more inhibitor of calmodulin (W13)	0.01
Cerebellum + *L. brevis* CD2 (5 micrograms)	0.72
Cerebellum + *L. brevis* CD2 + L-NAME	0.8
Cerebellum + *L. brevis* CD2 + EGTA	0.74
Cerebellum + *L. brevis* CD2 + W13	0.76
Cerebellum + *L. fermentum* (30 microgrammes)	0.38
Cerebellum + *L. fermentum* + L-NAME	0.4
Cerebellum + *L. fermentum* + EGTA	0.4
Cerebellum + *L. fermentum* + W13	0.39
Untreated macrophages	0
Macrophages + LPS + IFN	0.32
Macrophages + LPS + IFN + L-NAME	0
Macrophages + *L. brevis* CD2 (5 micrograms)	0.76
Macrophages + *L. brevis* CD2 + L-NAME	0.78
Macrophages + LPS + IFN + *L. brevis* CD2	0.81
Macrophages + LPS + IFN + *L.brevis* CD2 + L-NAME	0.82
Macrophages + *L. fermentum* (30 micrograms)	0.4
Macrophages + *L. fermentum* + L-NAME	0.41
Macrophages + LPS + IFN + *L. fermentum*	0.42
Macrophages + LPS + IFN + *L. fermentum* + L-NAME	0.4

It seems to be evident that not all the bacteria have an enzymatic activity in respect of arginine deiminase of a significant level for the purposes of the present invention (Table 2) and that the strains which are endowed therewith inhibit both constituent NOS and inducible NOS, as is confirmed by the persistent presence of high values of citrulline even in the presence of specific inhibitors of the two types of NOS (Table 3).

Example 3

4 patients were treated, suffering from pouchitis, a non-specific inflammation of the ileal reservoir, which is most frequently complicated in the long term by the occurrence of ileo-ano-anastomosis for ulcerative colitis. It has recently been suggested that pouchitis is the result of inflammatory NO-mediated damage. The subjects, all volunteers, were treated for 2 months with a lyophilised preparation of *L. brevis* CD2 at a concentration of $5x10^{10}$ CFU/gr, by mouth, at a dosage of 6 g/day. Before and after the treatment a biopsy sample was taken from the mucous membrane of the pouch, which was subjected to homogenisation and then to dosage of the citrulline by means of analysis of the conversion of radio-marked arginine into citrulline.

Table 4

Effect of the treatment with CD2 on the activity of inducible nitric oxide synthase in intestinal biopsies of patients with pouchitis

	Citrulline (pmol/mg proteins/min)	
Patients	T0	T1
1	2.95	0.89
2	1.15	0.56
3	0.56	0.5
4	0.47	0.28
5	0.7	0.5

The treatment with CD2 afforded a significant reduction in the levels of activity of inducible nitric oxide synthase.

CLAIMS

1. A strain of bacteria characterised by an activity of arginine deiminase, expressed as picomols citrulline/mg bacterial proteins/min, of not less than 0.1, and their descendants, mutants and derivatives which retain said activity.

2. A strain according to claim 1 selected from lactic bacteria.

3. A strain according to claim 2 selected from *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus casei*.

4. A strain according to claim 3 which is the strain *Lactobacillus brevis* CD2 deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany, under the access number DSM 11988.

5. Use of bacteria endowed with arginine deiminase to prepare a pharmaceutical or dietetic composition capable of inducing apoptosis and/or reducing an inflammatory reaction and/or producing a curative effect in intestinal malabsorption and pancreatic insufficiency with modulation of the metabolic and/or nutritional state of the subject to be treated.

6. Use according to claim 5 wherein the bacteria are Gram-positive bacteria.

7. Use according to claim 5 wherein the bacteria are Gram-negative bacteria.

8. Use according to claim 5 wherein the bacteria are lactic bacteria.

9. Use according to claim 8 wherein the lactic bacteria are selected from the species *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus casei*.

10. Use according to claim 9 wherein the lactic bacteria belong to any one of the strains according to claim 1 or claim 2.

11. Use according to claim 9 wherein the lactic bacteria belong to the strain *Lactobacillus brevis* CD2 deposited with the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany, under the access number DSM 11988.

12. Use according to any one of claims 5 to 11 in the prevention and treatment of tumours in general and in particular colon-rectal cancer, cancer of the liver, gliomae, neuroblastomae, squamocellular oral carcinoma, lymphoid tumours, cancer of the prostate gland, cancer of the bladder, cancer of the breast, cancer of the pleura and the peritoneum, serious myasthenia, systemic lupus erythematosus, and other auto-immune diseases including those of the thyroid, diseases characterised by acute and/or chronic inflammatory processes, bronchial asthma, intestinal inflammatory diseases, gastrites, duodenites, gastric ulcers, duodenal ulcers, pneumonias and pleurisies, infections from adenovirus, baculovirus and in general supported by a viral agent, diseases characterised by acute and/or chronic inflammatory and/or degenerative processes of the central and/or peripheral nervous system, pancreatites, endomyocardites and ischaemic damage (myocardiac, retinal, cerebral and renal), urolithiases, nephrocalcinoses, hyperoxaluria, hyperphosphaturia, nephroalteration in the systemic and/or district arterial and/or venous pressure such as portal hypertension, vaginoses and vaginites, procto-haemerrhoidal inflammations, prostates, sinusites and otites, conjunctivites,

gingivites, periodontopathy, anaphylactic phenomena and immunodeficiencies.

13. Use according to any one of claims 5 to 12 wherein the bacteria are administered individually or in association with each other or with *Lactobacillus acidophilus*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus catenaforme*, *Lactobacillus cellobiosus*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus jensenii*, *Lactobacillus leichmanii*, *Lactobacillus minutus*, *Lactobacillus plantarum*, *Lactobacillus rogosae*, *Lactobacillus salivarius*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, *Bifidobacterium eriksonii*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium plantarum*, *Bifidobacterium pseudo-catenulatum*, *Bifidobacterium pseudolongum*, *Streptococcus lactis*, *Streptococcus raffinolactis*, *Streptococcus thermophilus*, *Acidaminococcus fermenta*, *Cytophaga fermentans*, *Rhodoferax fermentans*, *Cellulomonas fermentans* and *Zymomonas mobilis* and/or in association with arginine deiminase, sphingomyelinase or other enzymes, with cortisone, anti-inflammatory, immuno-modulant, cytostatic, immunological, endocrinological, vascular, anaesthetic, and vasodilatory drugs, growth factors, cytokines, ceramides, vitamins and minerals, lipids, amino acids and carbohydrates, formulations for enteric use and dietetic, prebiotic or probiotic supplements, and with excipients commonly used in the pharmaceutical industry or in the foodstuffs/dietetic field.

14. Use according to any one of claims 5 to 13 wherein the bacteria are lyophilised or sonicated.

15. A pharmaceutical composition which can be administered enterally, topically, rectally, nasally or parenterally, to induce

apoptosis and/or reduce an inflammatory reaction, which comprises a quantity of bacteria endowed with arginine deiminase effective to induce apoptosis and/or reduce an inflammatory reaction and/or produce a curative effect in intestinal malabsorption and in pancreatic insufficiency with modulation of the metabolic and/or nutritional state of the subject to be treated.

16. A composition according to claim 15 wherein the bacteria are Gram-positive bacteria.

17. A composition according to claim 15 wherein the bacteria are Gram-negative bacteria.

18. A composition according to claim 15 wherein the bacteria are lactic bacteria.

19. A composition according to claim 18 wherein the lactic bacteria are selected from the species *Lactobacillus brevis*, *Lactobacillus fermentum* and *Lactobacillus casei*.

20. A composition according to claim 19 wherein the lactic bacteria belong to any one of the strains according to claims 1, 2 or 3.

21. A composition according to claim 19 wherein the lactic bacteria belong to the strain *Lactobacillus brevis* CD2 deposited with the DSM - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Brunswick, Germany, under the access number DSM 11988.

22. A composition according to any one of claims 15 to 21 wherein the bacteria are lyophilised or sonicated.

23. A composition according to any one of claims 15 to 22 wherein the concentration of the bacteria is from $1x10^{1}$ CFU to $1x10^{13}$ CFU per gram of composition.

24. A composition according to any one of claims 15 to 23 for use in the prevention and treatment of tumours in general and in particular colon-rectal cancer, cancer of the liver, gliomae, neuroblastomae, squamocellular oral carcinoma, lymphoid tumours, cancer of the prostate gland, cancer of the bladder, cancer of the breast, cancer of the pleura and the peritoneum, serious myasthenia, systemic lupus erythematosus, and other auto-immune diseases including those of the thyroid, diseases characterised by acute and/or chronic inflammatory processes, bronchial asthma, intestinal inflammatory diseases, gastrites, duodenites, gastric ulcers, duodenal ulcers, pneumonias and pleurisies, infections from adenovirus, baculovirus and in general supported by a viral agent, diseases characterised by acute and/or chronic inflammatory and/or degenerative processes of the central and/or peripheral nervous system, pancreatites, endomyocardites and ischaemic damage (myocardiac, retinal, cerebral and renal), urolithiases, nephrocalcinoses, hyperoxaluria, hyperphosphaturia, nephroalteration in the systemic and/or district arterial and/or venous pressure such as portal hypertension, vaginoses and vaginites, procto-haemerrhoidal inflammations, prostates, sinusites and otites, conjunctivites, gingivites, periodontopathy, anaphylactic phenomena and immunodeficiencies.

25. A composition according to any one of claims 15 to 24 wherein the bacteria are administered individually or in association with each other or with *Lactobacillus acidophilus*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus catenaforme*, *Lactobacillus cellobiosus*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus delbrueckii*, *Lactobacillus jensenii*, *Lactobacillus leichmanii*, *Lactobacillus minutus*, *Lactobacillus plantarum*,

Lactobacillus rogosae, *Lactobacillus salivarius*, *Bifidobacterium adolescentis*, *Bifidobacterium angulatum*, *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, *Bifidobacterium eriksonii*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Bifidobacterium plantarum*, *Bifidobacterium pseudo-catenulatum*, *Bifidobacterium pseudolongum*, *Streptococcus lactis*, *Streptococcus raffinolactis*, *Streptococcus thermophilus*, *Acidaminococcus fermenta*, *Cytophaga fermentans*, *Rhodoferax fermentans*, *Cellulomonas fermentans* and *Zymomonas mobilis* or in association with arginine deiminase, sphingomyelinase or other enzymes, with cortisone, anti-inflammatory, immuno-modulant, cytostatic, immunological, endocrinological, vascular, anaesthetic, and vasodilatory drugs, growth factors, cytokines, ceramides, vitamins and minerals, lipids, amino acids and carbohydrates, formulations for enteric use and dietetic, prebiotic or probiotic supplements, and with excipients commonly used in the pharmaceutical industry or in the foodstuffs/dietetic field.

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/IT 98/00275

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/78 A61K38/50 //C12R1/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MANCA DE NADRA M ET AL: "Isolation and properties of Arginine Deiminase in Lactobacillus buchneri NCDO110" J. APPL. BIOCHEM., vol. 6, 1984, pages 184-187, XP002092818	1-4
Y	see table 1 ---	5-25
Y	TAKAKU H ET AL: "Anti-tumor activity of arginine deiminase from mycoplasma arginini and its growth-inhibitory mechanism" JPN. J. CANCER RES., vol. 86, no. 9, September 1995, pages 840-846, XP002092819 cited in the application see abstract --- -/--	5-25

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 February 1999	23/02/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lonnoy, O

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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/IT 98/00275

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 196 195 A (GRIFFITH OWEN W) 23 March 1993 see column 3, line 11 - line 41; claim 2	5-25
X	WO 95 32720 A (HYBRID SCIENT PTY LTD ;WINN GREGORY MURRAY (AU); BORUSHEK JOHN BEN) 7 December 1995 see the whole document in general, and claim 17 in particular	5-25
X	EP 0 508 701 A (QUEST INT) 14 October 1992 see the whole document	5-25
A	NARITA I ET AL: "L-Arginine may mediate the therapeutic effects of low protein diets" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 92, May 1995, pages 4552-4556, XP002092820 see abstract	
A	EP 0 414 007 A (NIPPON MINING CO) 27 February 1991	
A	MANCA DE NADRA M ET AL: "Arginine dehydrolase pathway in Lactobacillus buchneri: a review" BIOCHIMIE, vol. 70, 1988, pages 367-374, XP002092821 see table 1	

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

...ternational application No.
PCT/IT 98/00275

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.: not applicable
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims: it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Claims Nos.: not applicable

In view of the extremely broad claims (i.e. claims 5, 15, and dependent claims thereof), the search was executed with due regard to the PCT Search Guidelines (PCT/GL/2), C-III, paragraph 2.1, 2.3 read in conjunction with 3.7 and rule 33.3 PCT, i.e. particular emphasis was put on the inventive concept, as illustrated by the use of bacteria endowed with arginine deiminase in pharmaceutical or dietetic compositions capable of decreasing arginine availability, and as illustrated in example 3. The international search was, in so far as possible and reasonable, complete in that it covered the entire subject-matter to which the claims are directed.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ional Application No
PCT/IT 98/00275

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